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Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus* reverse transcriptase

(antiviral chemotherapy/acquired immunodeficiency syndrome/thymidine kinase/thymidylate kinase)

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ABSTRACT **The thymidine analog 3′-azido-3′-deoxythymidine (BW A509U, azidothymidine) can inhibit human immunodeficiency virus (HIV) replication effectively in the 50–500 nM range [Mitsuya, H., Weinhold, K. J., Furman, P. A., St. Clair, M. H., Nusinoff-Lehrman, S., Gallo, R. C., Bolognesi, D., Barry, D. W. & Broder, S. (1985) *Proc. Natl. Acad. Sci. USA* 82, 7096–7100]. In contrast, inhibition of the growth of uninfected human fibroblasts and lymphocytes has been observed only at concentrations above 1 mM. The nature of this selectivity was investigated. Azidothymidine anabolism to the 5′-mono-, -di-, and -triphosphate derivatives was similar in uninfected and HIV-infected cells. The level of azidothymidine monophosphate was high, whereas the levels of the di- and triphosphate were low (≤5 μM and ≤2 μM, respectively). Cytosolic thymidine kinase (EC 2.7.1.21) was responsible for phosphorylation of azidothymidine to its monophosphate. Purified thymidine kinase catalyzed the phosphorylations of thymidine and azidothymidine with apparent K_m values of 2.9 μM and 3.0 μM. The maximal rate of phosphorylation with azidothymidine was equal to 60% of the rate with thymidine. Phosphorylation of azidothymidine monophosphate to the diphosphate also appeared to be catalyzed by a host-cell enzyme, thymidylate kinase (EC 2.7.4.9). The apparent K_m value for azidothymidine monophosphate was 2-fold greater than the value for dTMP (8.6 μM vs. 4.1 μM), but the maximal phosphorylation rate was only 0.3% of the dTMP rate. These kinetic constants were consistent with the anabolism results and indicated that azidothymidine monophosphate is an alternative-substrate inhibitor of thymidylate kinase. This conclusion was reflected in the observation that cells incubated with azidothymidine had reduced intracellular levels of dTTP. IC_{50} (concentration of inhibitor that inhibits enzyme activity 50%) values were determined for azidothymidine triphosphate with HIV reverse transcriptase and with immortalized human lymphocyte (H9 cell) DNA polymerase α. Azidothymidine triphosphate competed about 100-fold better for the HIV reverse transcriptase than for the cellular DNA polymerase α. The results reported here suggest that azidothymidine is nonselectively phosphorylated but that the triphosphate derivative efficiently and selectively binds to the HIV reverse transcriptase. Incorporation of azidothymidylate into a growing DNA strand should terminate DNA elongation and thus inhibit DNA synthesis.**

Recently, Mitsuya *et al.* (1) reported that 3′-azido-3′-deoxythymidine (azidothymidine) was a selective and potent inhibitor of human T-cell lymphotropic virus type III (HTLV-III, now called human immunodeficiency virus, HIV) replication in several different lymphocyte cultures. Many other thymidine analogs are known to inhibit other viruses in cell culture with varying degrees of selectivity (2, 3). Selectivity of activation can result from increased levels of cytosolic or virus-encoded thymidine kinase in the infected cells. Although the monophosphate of some analogs can inhibit thymidylate synthase, a more common target for interaction is the virus-encoded DNA polymerase. Selectivity at this level results from a higher affinity of the analog triphosphate for the viral polymerase than for cellular polymerase(s). Incorporation of the analog monophosphate into viral DNA may be a critical event for some analogs (2).

Whether any of these modes of selectivity or targets of inhibition pertain to the selective inhibition of HIV by azidothymidine was not known. The retroviruses are not known to code for a thymidine kinase, and the viral polymerase for which they code (RNA-directed DNA polymerase, reverse transcriptase, EC 2.7.7.49) is catalytically quite distinct from cellular DNA polymerases (4, 5). An additional aspect to the mode of action of azidothymidine is that the 3′-azido group should cause termination of DNA elongation if the nucleoside moiety were incorporated into DNA.

Here we show that azidothymidine is phosphorylated to its 5′-triphosphate derivative by cellular enzymes of the thymidine-phosphorylation pathway [thymidine kinase (EC 2.7.1.21) and thymidylate kinase (EC 2.7.4.9)]. Further, we show that azidothymidine 5′-triphosphate selectively competes for the viral reverse transcriptase, suggesting that the reverse transcriptase of HIV can be a specific target for antiviral chemotherapy.

MATERIALS AND METHODS

Cells and Viruses. Human foreskin fibroblasts (MRHF) and human diploid embryonic lung fibroblasts (MRC5) were obtained from Whittaker M.A. Bioproducts (Walkersville, MD) and the American Type Culture Collection, respectively. Both cell cultures were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum

Abbreviations: HIV, human immunodeficiency virus; PBL, peripheral blood lymphocyte.

*The Executive Committee of the International Committee on Taxonomy of Viruses (ICTV) has endorsed the name human immunodeficiency virus (to be abbreviated HIV) recently proposed by a majority of the members of a study group of ICTV as appropriate for the retrovirus isolates implicated as the causative agents of acquired immunodeficiency syndrome [(1986) *Science* **232**, 1486, and *Nature (London)* **321**, 644].

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(Sterile Systems, Logan, UT) and 2 mM L-glutamine. Vero cells were grown in Eagle's MEM supplemented with 5% newborn calf serum (Whittaker M.A. Bioproducts) and 5% fetal bovine serum. Clone H9, an $OKT4^+$ cell line permissive for HIV (6), was obtained from B. Hampar (National Cancer Institute, Frederick Cancer Center) and grown in RPMI 1640 medium containing 20% fetal bovine serum. FG-10 cells (7) were maintained in McCoy's 5-A medium supplemented with 10% fetal bovine serum. Peripheral blood lymphocytes (PBLs), first stimulated for 48 hr with phytohemagglutinin (PHA; Difco), were cultured in RPMI 1640 containing 10% delectinated interleukin 2 (Cellular Products, Buffalo, NY) and supplemented with 10% fetal bovine serum. HIV was obtained from the culture fluid of HIV-producing H9 cells (6) kindly provided by R. Gallo (National Cancer Institute). The number of viable cells was determined by dye exclusion with erythrosin B.

Chemicals. Azido[5'-^{3}H]thymidine was synthesized in these laboratories by J. Hill and G. A. Freeman by unpublished procedures. Final purification (>99.9% homogeneous) was achieved by chromatography through a C_{18} reversed-phase column with a 25–50% methanol gradient. The mono-, di-, and triphosphates of azidothymidine were prepared from azidothymidine by published methods (8–10). The phosphorylated derivatives of azidothymidine were isolated in these laboratories by W. Miller (11). The monophosphate of [^{3}H]azidothymidine was synthesized with H9 cellular thymidine kinase (12) and was purified with a Mono Q column (Pharmacia) at pH 2.8. Other chemicals were as used previously (12).

Cell Growth and Cytotoxicity Assays. The effect of azidothymidine on cell growth was measured in a 72-hr growth-inhibition assay described previously (13). Cell cytotoxicity using clone H9 cells and phytohemagglutinin-stimulated PBLs was determined by erythrosin B exclusion after incubation with various concentrations of azidothymidine for 72 hr at 37°C. H9 cells and PBLs were cultured in medium containing various concentrations of drug at 2×10^5 and 5×10^5 cells per ml, respectively.

Enzyme Preparations. Cell extracts were $10,000 \times g$ supernatants of frozen–thawed (three cycles) H9 cell pellets in four volumes of extraction buffer (10 mM Tris·HCl, pH 7.5/10 mM KCl/1 mM $MgCl_2$/1 mM dithiothreitol). Cytosolic thymidine kinase and thymidylate kinase from H9 cells were purified by affinity chromatography as described (12). Enzyme was stored in elution buffer and desalted by size-exclusion chromatography just before use. Less than 1% dTMP- or dTDP-phosphohydrolyzing activity was detected with either enzyme.

HIV reverse transcriptase was purified by a modification of the procedure of Abrell and Gallo (14). In brief, 750 ml of culture fluid harvested from HIV-infected H9 cells was centrifuged at 18,000 rpm for 90 min in an R19 rotor (Beckman) to pellet virus. Enzyme was extracted by incubating the virus pellet in buffer A [50 mM Tris·HCl, pH 7.9/0.25% Nonidet P-40/20 mM dithiothreitol/5% (vol/vol) glycerol] containing 1 mM EDTA, 500 mM KCl, and 0.5% deoxycholate. The enzyme was partially purified by passing the extract through a DEAE-cellulose column (3×10 cm) previously equilibrated with buffer A. Fractions containing enzyme activity were dialyzed against buffer B (50 mM Tris·HCl, pH 7.9/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol/20% glycerol) and were further purified by phosphocellulose chromatography. The peak fractions were pooled and dialyzed against buffer B containing 50% glycerol. To the dialyzed enzyme, bovine serum albumin was added to give a final concentration of 1 mg/ml. The enzyme was characterized as HIV reverse transcriptase based on its cation, salt, pH, and template requirements (5). Cellular DNA polymerase α was purified as described (15).

Enzyme Assays. Phosphorylation of thymidine, azidothymidine, or their monophosphates was measured at 37°C by a DEAE-paper disk method (16) or by measuring the conversion of the substrate to product by excision and scintillation counting of the appropriate portions of polyethylenimine thin-layer plates (12, 16) after development with solvent I (50% methanol) or solvent II (0.3 M sodium formate, pH 3.4/50% methanol). Standard reaction mixtures for nucleoside phosphorylation contained 50 mM Tris·HCl (pH 7.5), 5 mM ATP·Mg, 0.1 mM [^{14}C]thymidine or [^{3}H]azidothymidine (90 and 700 cpm/pmol), and enzyme. For measurements of nucleoside monophosphate phosphorylations, [^{14}C]dTMP (90 cpm/pmol) or [^{3}H]azidothymidine monophosphate (700 cpm/pmol) was substituted for the radiolabeled nucleosides. Enzyme activity was proportional to enzyme concentration and time of reaction. The apparent K_m (K'_m) or K_{is} values were determined as described (12). One unit of enzyme activity was defined as the amount of enzyme that would convert 1 pmol of thymidine or dTMP to its phosphorylated product per minute under the above standard conditions.

The purified HIV reverse transcriptase was assayed using reaction conditions similar to those of Abrell and Gallo (14) and adapted to the DEAE-paper disk assay (15). Reaction mixtures (150 μl) contained 50 mM Tris·HCl (pH 7.3); 100 mM KCl; 5 mM $MgCl_2$; 1 mM dithiothreitol; 80 μM each dATP, dCTP, and dGTP; and 5.6 μM [^{3}H]dTTP (4800 cpm/pmol), unless otherwise stated. The template concentration was 20 μg/ml for poly(rA)·oligo(dT)$_{12-18}$ (Pharmacia P-L Biochemicals) and for activated calf thymus DNA. Cellular DNA polymerase α was assayed as described (15). IC_{50} values (concentration of inhibitor that inhibits enzyme activity by 50%) for HIV reverse transcriptase and H9 DNA polymerase α were calculated using the Probit computer program (17).

High-Performance Liquid Chromatography Analysis. Neutralized perchloric acid extracts of cells treated with 50 μM [^{3}H]azidothymidine were analyzed by HPLC as described (18). Azidothymidine nucleotides were separated by elution with a linear gradient of 0.015–1 M KH_2PO_4 (pH 3.5), developed over 110 min at a flow rate of 0.5 ml/min. Intracellular levels of deoxyribonucleoside triphosphates were quantitated as described (19).

Identification of Phosphorylated Products. Phosphorylated products obtained from cells or from phosphorylation of azidothymidine catalyzed by cell extracts were identified as 5'-phosphates of azidothymidine by treatment of the products with *Crotalus atrox* phosphodiesterase I (EC 3.1.4.1), which hydrolyzed the di- and triphosphates completely to the monophosphate, and then with *C. atrox* 5'-nucleotidase (EC 3.1.3.5) to produce the nucleoside analog. Subsequent cochromatography with azidothymidine through a C_{18} reversed-phase column (retention volume 5.8 ml in 30% methanol) confirmed that the analog was, in fact, azidothymidine.

RESULTS

Effects of Azidothymidine on Cell Growth and Viability. The effect of azidothymidine on cell growth varied with cell type (Table 1). Human fibroblasts and lymphocytes showed little inhibition of growth except at very high concentrations ($ID_{50} \geq 1000$ μM), whereas epithelial cells of murine and primate origin were somewhat more sensitive (ID_{50} = 30 and 170 μM, respectively). This variation in sensitivity to azidothymidine appeared to correlate with levels of azidothymidine 5'-triphosphate in these cells (unpublished observations). Cytotoxicity assays with H9 cells and PBLs also required high concentrations of azidothymidine ($\geq$500 μM) to achieve the 50% end point (Table 1).

Anabolism of Azidothymidine. The nucleotide profile revealed the presence of radioactivity in the mono-, di-, and

Table 1. Effect of azidothymidine on rate of cell growth and cell viability

Cells	Derivation	ID_{50}*, μM	
		Growth	Viability
MRHF	Human foreskin	3500	ND
MRC5	Human embryonic lung	1700	ND
H9	Immortalized human T cells	1000	>1000†
PBLs	Human peripheral blood	ND	500
Vero	African green monkey kidney	170	ND
FG-10	Mouse 3T3 subline	30	ND

ND, not determined.
*Concentration of inhibitor that reduces cell growth or cell viability by 50% following exposure to the inhibitor for 72 hr.
†Azidothymidine at 1000 μM reduced H9 cell viability 30%.

triphosphate regions of the chromatogram (Fig. 1). Occasionally, minor peaks at about 19 min (<1% of total) and 47 min (<0.3% of total) were observed. The material at about 17 min was azidothymidine and was calculated to be less than or equal to the concentration of azidothymidine in the medium. The major derivatives were identified as 5'-phosphates of azidothymidine (see *Materials and Methods*). High concentrations of azidothymidine monophosphate were detected in the uninfected and the HIV-infected H9 cells (Fig. 1 and Table 2), whereas the levels of the diphosphate and triphosphate were low. By 24 hr these phosphorylated derivatives had accumulated maximally. Even though the level of azidothymidine monophosphate that accumulated during a 24-hr exposure to azidothymidine decreased somewhat throughout the replication cycle of the virus, no significant differences were noted in the levels of the di- and triphosphate derivatives. Increasing the time that the cells were exposed to the drug did not result in higher levels of phosphorylated derivatives.

The decrease in levels of the phosphorylated derivatives of azidothymidine, after removal of the drug from the incubation medium, was measured during peak virus replication (days 5–6). HIV-infected cells were incubated for 24 hr in medium containing 50 μM [^{3}H]azidothymidine, after which the cells were washed and the incubation was continued in drug-free medium. Perchloric acid extracts for HPLC analysis were then prepared at various times during the incubation. Under these conditions, the levels of the phosphorylated derivatives of azidothymidine declined rapidly with time (Table 3). However, 4 hr after removal of azidothymidine, the intracellular concentration of the triphosphate was still ≈1 μM.

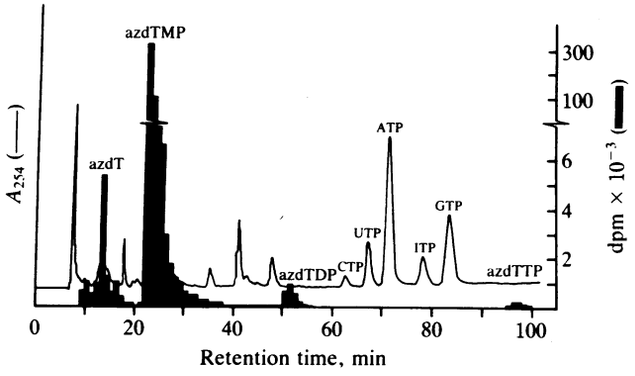

FIG. 1. HPLC profile of an extract of HIV-infected H9 cells incubated with 50 μM [^{3}H]azidothymidine for 24 hr at day 3 of infection. azdT, azidothymidine; azdTMP, azidothymidine 5'-monophosphate; azdTDP, azidothymidine 5'-diphosphate; azdTTP, azidothymidine 5'-triphosphate.

Table 2. Anabolism of azidothymidine in uninfected and HIV-infected H9 cells

Cells	Concentration, pmol per 10^6 cells (μM)		
	Monophosphate	Diphosphate	Triphosphate
Uninfected	660 (790)	3.5 (4.2)	1.5 (1.8)
Infected			
24 hr	460 (560)	2.6 (3.1)	0.9 (1.1)
Days 3–4	340 (410)	2.0 (2.4)	1.0 (1.3)
Days 6–7	170 (200)	4.4 (5.3)	1.7 (2.0)
Days 9–10	170 (210)	4.7 (5.6)	0.9 (1.1)

H9 cells were infected with HTLV-III_B (HIV derived from a pool of American patients) at a multiplicity of infection of 10,000 particles per cell. Cells were incubated with 50 μM [^{3}H]azidothymidine (424,000 dpm/nmol) for 24 hr either during infection or at the indicated days during the infection cycle. Concentrations (μM, values in parentheses) of the phosphorylated derivatives of azidothymidine were calculated using a packed cell volume of 8.5 × 10^8 cells/ml of packed cells.

Phosphorylation of Azidothymidine with Cell Extracts and Purified Enzymes. The structural similarity of azidothymidine to thymidine suggested that thymidine kinase might catalyze the phosphorylation of the analog. With cell extracts, thymidine and azidothymidine (100 μM) were phosphorylated at rates of 26 and 17 pmol/min per 10^6 viable cells, respectively. There was no apparent virus stimulation of these activities during a 10-day course of HIV infection.

Phosphorylation of azidothymidine catalyzed by cell extracts was totally inhibited by thymidine; conversely, thymidine phosphorylation was inhibited by azidothymidine. This suggested that azidothymidine and thymidine phosphorylations were catalyzed by a single enzyme. Calculations from inhibition data, assuming an apparent K_m value for thymidine of 3 μM (20), indicated a K_i value for azidothymidine of about 2 μM. The values of relative phosphorylation rates and apparent K_m values from extracts were compared to values obtained with purified cytosolic thymidine kinase from H9 cells. Azidothymidine was phosphorylated with a maximal velocity 60% that of thymidine and with an apparent K_m value of 3.0 μM compared to a value of 2.9 μM for thymidine (Table 4). These phosphorylation rates and K_m values determined with the purified enzyme corresponded well with the values obtained with the cell extracts (see above). This correspondence supported the premise that azidothymidine phosphorylation is catalyzed by the thymidine kinase of the cell.

Phosphorylation of azidothymidine monophosphate to the diphosphate appeared to be catalyzed by thymidylate kinase. Phosphorylation of azidothymidylate catalyzed by extracts

Table 3. Levels of phosphate derivatives after azidothymidine removal

Time after removal, hr	Concentration, pmol per 10^6 cells (μM)		
	Monophosphate	Diphosphate	Triphosphate
0	820 (990)	5.5 (6.6)	6.0 (7.2)
0.5	300 (360)	6.0 (7.2)	4.3 (5.2)
1	190 (230)	1.3 (1.6)	1.6 (1.9)
2	42 (50)	0.7 (0.8)	1.4 (1.7)
4	13 (16)	0.6 (0.7)	0.8 (1.0)

H9 cells were infected with HTLV-III_B (10^4 particles per cell; see legend to Table 2) for 18 hr. At day 5 after infection, cells were incubated for 24 hr with 50 μM [^{3}H]azidothymidine (541,680 dpm/pmol). The [^{3}H]azidothymidine was then removed and the cultures were washed three times with 12 ml of prewarmed medium. Prewarmed medium lacking azidothymidine was added to the cultures. At the indicated times the cells were harvested and extracted, and the extracts were assayed as described in *Materials and Methods*.

Table 4. Kinetic values for the phosphorylation of azidothymidine and azidothymidylate

Enzyme	Substrate	K_m', μM	Relative V_{max}, %
Thymidine kinase	Thymidine	2.9 ± 0.5	100
	Azidothymidine	3.0 ± 0.3	60
Thymidylate kinase	Thymidylate	4.1 ± 0.9	100
	Azidothymidylate	8.6 ± 0.6	0.3

Purified thymidine kinase from the cytosol of H9 cells catalyzed the conversion of radiolabeled thymidine or azidothymidine to the 5'-monophosphate. Product was separated from substrate by thin-layer chromatography with solvent I. Apparent K_m (K_m') values were determined as described in *Materials and Methods*, with a 0.7–13 μM range of substrate concentration. The values for thymidylate kinase were determined as described in the legend to Fig. 2 and in the text.

of H9 cells was fully inhibited by the addition of dTMP (500 μM). Further evidence that azidothymidylate bound to dTMP kinase was obtained by measuring the effects of azidothymidylate on the phosphorylation of dTMP with purified dTMP kinase from cytosol extracts of H9 cells. The results indicated that azidothymidylate is a competitive inhibitor of dTMP phosphorylation, with a K_{is} value of 8.6 μM (Fig. 2). The apparent K_m value for dTMP (4.1 μM) was identical to that determined by Chen *et al.* (21) with enzyme from Vero cells.

Radiolabeled azidothymidylate was used to compare the rate of phosphorylation to that of dTMP. Its relative maximal velocity was very low (Table 4). In spite of the low rate, since azidothymidylate was a substrate for the dTMP kinase, its apparent K_m value is equal to the K_{is} value determined above (22). Because of the low K_m and low V_{max} values for azidothymidylate, it can be considered an effective alternative-substrate inhibitor of dTMP kinase.

Effect of Azidothymidine on Intracellular Deoxyribonucleoside Triphosphate Levels. In three separate experiments, intracellular dTTP levels were severely reduced when H9 cells were exposed to 50 μM azidothymidine (Table 5). In addition to the effect on dTTP levels, a large decrease in

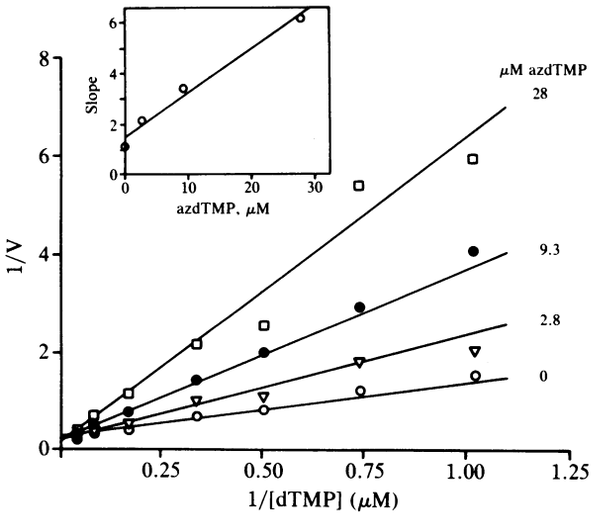

FIG. 2. Inhibition of thymidylate kinase by azidothymidylate (azdTMP). Purified cytosolic thymidylate kinase from H9 cells (150 units/ml) of reaction mixture) catalyzed the conversion of [^{14}C]dTMP to [^{14}C]dTDP. Reaction rates are expressed as pmol of dTDP formed per min. Thin-layer chromatography with solvent II was used to separate product from substrate. Other details were as described in *Materials and Methods*. The K_{is} value for azidothymidylate was determined from the replot of the slopes vs. azidothymidylate (azdTMP) concentration (*Inset*).

dCTP levels, an ≈50% decrease in dGTP levels, and a significant increase in dATP levels were observed. A similar trend was observed with HIV-infected H9 cells after exposure to 50 μM azidothymidine for 24 and 48 hr during the period of peak virus replication (days 5–7).

Azidothymidine Triphosphate Inhibits [^{3}H]dTMP Incorporation Catalyzed by HIV Reverse Transcriptase. The ability of azidothymidine triphosphate to bind to the HIV reverse transcriptase and DNA polymerase α of H9 cells was assessed by measuring the inhibition of [^{3}H]dTMP incorporation into a primer-template by azidothymidine triphosphate. The reverse transcriptase was much more sensitive to inhibition than was the DNA polymerase α of H9 cells (Fig. 3). The IC_{50} values for the viral reverse transcriptase were 0.7 μM with poly(rA)·oligo(dT)$_{12-18}$ and 2.3 μM with activated calf thymus DNA as primer-templates. In contrast, an IC_{50} value of 260 μM was determined for azidothymidine triphosphate with the H9 DNA polymerase α when activated calf thymus DNA was used as primer-template. The kinetics of inhibition of the HIV reverse transcriptase by azidothymidine triphosphate were also determined using standard Lineweaver–Burk plots. The results of these experiments (data not shown) indicated that azidothymidine triphosphate competed with dTTP for the enzyme and has a K_{is} value of 0.04 ± 0.002 μM. The enzyme had an apparent K_m of 2.8 ± 0.25 μM for dTTP. In addition, the K_i values for the α and β DNA polymerases of H9 cells were 230 μM and 70 μM, respectively, when activated calf thymus DNA was used as template (data not shown).

DISCUSSION

The thymidine analog 3'-azido-3'-deoxythymidine is a potent inhibitor of HIV replication *in vitro* (1) and shows little effect on the growth of cultured human fibroblasts and lymphocytes (Table 1). The *in vitro* "therapeutic index" (concentration that inhibits cell growth 50% divided by the concentration that inhibits virus replication 50%) for azidothymidine with the target H9 cells or PBLs was ≥10^4. These results suggest that, in these cells, azidothymidine is a highly selective inhibitor of HIV replication.

Anabolism and enzyme experiments showed that a virus-encoded or virus-induced enzyme played no role in the anabolic activation of azidothymidine. This analog was a good substrate for the cellular thymidine kinase and was phosphorylated as efficiently as thymidine. This observation contrasts with the activation of acyclovir by a virus-encoded rather than cellular thymidine kinase (16).

The second phosphorylation step for azidothymidine was catalyzed by the cellular dTMP kinase. Contrary to the case with thymidine kinase, the dTMP kinase phosphorylated azidothymidylate only inefficiently. This was consistent with the idea that dTMP kinase catalyzed the rate-limiting step in the anabolism of azidothymidine to the triphosphate.

Because the levels of azidothymidylate in azidothymidine-treated H9 cells were so high, we cannot rule out the

Table 5. Intracellular deoxyribonucleoside triphosphate levels in H9 cells exposed to azidothymidine

Exposure time, hr	pmol per 10^6 cells			
	dCTP	dTTP	dATP	dGTP
0	20 ± 4.2	37 ± 19	69 ± 17	52 ± 34
24	1.0 ± 0.6	7.8 ± 1.0	150 ± 48	17 ± 7
48	0.6 ± 0.1	2.3 ± 0.4	260 ± 170	27 ± 19
72	0.9 ± 0.2	2.0 ± 1.0	200 ± 30	19 ± 19

H9 cells were exposed to 50 μM azidothymidine for 24, 48, or 72 hr. Cells were extracted and the extracts were assayed as described (19). Values are means ± SD from 3 separate experiments.

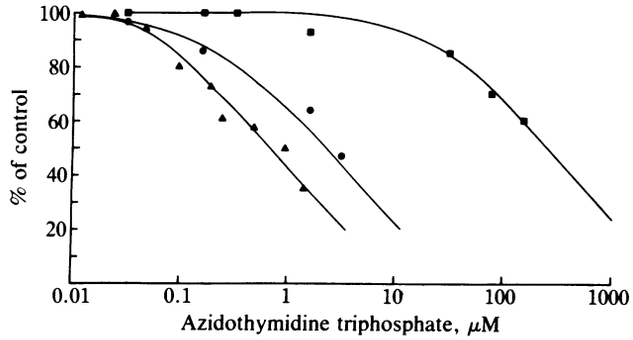

FIG. 3. Inhibition of HIV reverse transcriptase and H9 DNA polymerase α by azidothymidine triphosphate. Viral and cellular enzymes were isolated and purified as described (14, 15). Reactions were started by adding purified HIV reverse transcriptase. Reverse transcriptase was assayed using poly(rA)·oligo(dT)$_{12-18}$ (▲) or activated calf thymus DNA (●) as template. H9 cell DNA polymerase α was assayed using activated calf thymus DNA as template (■).

possibility that this form of the drug may also play a role in the inhibition of HIV. Preliminary results suggest that high levels of azidothymidylate (≥500 μM) inhibit the HIV reverse transcriptase but not the H9 DNA polymerase α (unpublished data). Inhibition of thymidylate synthase by azidothymidylate does not seem to play a role in the mechanism of viral inhibition, since the thymidylate synthase from calf thymus was not inhibited by 2 mM azidothymidylate (Inderjit Dev, personal communication).

Azidothymidine triphosphate inhibited HIV reverse transcriptase ≈100 times better than it inhibited the H9 polymerase α, with activated calf thymus DNA as the template. When transcription of the viral RNA template was mimicked by using poly(rA)·oligo(dT)$_{12-18}$, the reverse transcriptase was 300 times more sensitive to inhibition.

Because the levels of azidothymidine triphosphate were low in HIV-infected cells incubated with azidothymidine, the question can be raised whether there is enough triphosphate present to bind to and possibly be a substrate for the reverse transcriptase. In these studies, intracellular levels of 1–7 μM azidothymidine triphosphate were observed. Preliminary kinetic data with the HIV reverse transcriptase suggest that these levels of triphosphate in the infected cells are 25- to 160-fold greater than the K_{is} (0.04 μM). Further, when azidothymidine was removed from infected cultures, the analog triphosphate persisted at concentrations 25 times the K_i for the reverse transcriptase for at least 4 hr during peak virus replication.

Exposure of cells to azidothymidine caused a reduction in the intracellular level of dTTP. This effect may play a role in the inhibition of HIV DNA synthesis, because reducing the level of the competing substrate, dTTP, would facilitate the binding of azidothymidine triphosphate to the reverse transcriptase. In addition to a role in the antiviral effect of azidothymidine, the changes in the intracellular levels of the deoxynucleoside triphosphates may play some role in the cytotoxicity observed in the 72-hr growth studies.

The data indicate that azidothymidine is anabolized as a thymidine analog in the first two phosphorylation steps and that these are catalyzed by cellular enzymes. Azidothymidine triphosphate preferentially interacts with the HIV reverse transcriptase. Preliminary evidence also suggests that azidothymidine triphosphate can serve as an alternative substrate for the reverse transcriptase and that incorporation of azidothymidylate into a DNA primer-template results in chain termination (23). This highly selective incorporation into and chain termination of viral DNA synthesis via the reverse transcriptase appears to explain the high selectivity of azidothymidine for HIV compared with normal host cells.

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